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Early immune responses accompanying human asymptomatic Ebola infections

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SUMMARY

In a recent study we identified certain asymptomatic individuals infected by Ebola virus (EBOV) who mounted specific IgG and early and strong inflammatory responses. Here, we further characterized the primary immune response to EBOV during the course of asymptomatic infection in humans. Inflammatory responses occurred in temporal association with anti-inflammatory phase composed by soluble antagonist IL-1RA, circulating TNF receptors, IL-10 and cortisol. At the end of the inflammatory process, mRNA expression of T-cell cytokines (IL-2 and IL-4) and activation markers (CD28, CD40L and CTLA4) was up-regulated, strongly suggesting T-cell activation. This T-cell activation was followed by EBOV-specific IgG responses (mainly IgG3 ang IgG1), and by marked and sustained up-regulation of IFNγ, FasL and perforin mRNA expression, suggesting activation of cytotoxic cells. The terminal down-regulation of these latter markers coincided with the release of the apoptotic marker 41/7 NMP in blood and with the disappearance of viral RNA from PBMC, suggesting that infected cells are eliminated by cytotoxic mechanisms. Finally, RT-PCR analysis of TCR-Vβ repertoire usage showed that TCR-Vβ12 mRNA was never expressed during the infection. Taken together, these findings improve our understanding about immune response during human asymptomatic Ebola infection, and throw new light on protection against Ebola virus.

Keywords Ebola asymptomatic immunity cytokine TCR

INTRODUCTION

Ebola virus (EBOV) belongs to the *Filoviridae*-family, and consists of the Zaire, Sudan, Reston and Côte d'Ivoire subtypes [1]. EBOV possesses a non segmented negative-stranded RNA genome, 19 kb in lenght, which codes for seven structural proteins: nucleoprotein (NP), virion structural protein (VP) VP35, VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L) [2].

Serial data on the immune responses and pathogenesis of human Ebola haemorrhagic fever (EHF) are difficult to obtain: the most extensive studies are limited to IgM and IgG assays [3]. Experimental studies have shed some light on the pathogenesis on the infection [4–6], but there are no data on cellular responses. Given the inefficacy of passive immunization with immune plasma in primates and humans, humoral responses alone are unlikely to account for recovery or secondary protection [7,8]. *In vitro* and *in vivo* studies have shown that filoviruses can infect and propagate in macrophagic and endothelial cells [9–12]. During recent Ebola outbreaks in Gabon, we found that fatal outcome was associated with a suboptimal humoral response, no specific IgG production, and early activation of peripheral blood mononuclear cells (PBMC), followed by extensive intravascular apoptosis [13]. In contrast, survival was associated with early emergence of specific humoral responses, and tightly regulated activation of cytotoxic cells that coincided with clearance of viral antigens from blood.

Recently, we identified several individuals with documented close contact with symptomatic EHF patients who nonetheless remained asymptomatic and mounted IgM and IgG responses to the NP and VP40 EBOV proteins [14]. These asymptomatic individuals were infected by EBOV at a low level over several days. This first description of asymptomatic replicative Ebola infection raised questions about the mechanism of protection. As symptomatic and asymptomatic subjects were infected by the same viral strain (as suggested by partial genotyping), the protective mechanism may have involved the transient and high circulating levels of proinflammatory cytokines (IL-1β, TNFα, IL-6, MCP and, MIP-1α/β) observed in asymptomatics early during the infection (5–7 days after the first putative infectious contact).

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In the present study, we investigated cellular immune responses in seven asymptomatic individuals from 7 to 23 days after exposure to the virus. We showed that individuals asymptomatically infected with EBOV mounted a strong inflammatory response in temporal association with a counteract anti-inflammatory one. This inflammatory process is then followed by a well regulated T cell response leading to the generation of EBOV-specific IgG1 and IgG3 subclasses responses and to marked and sustained activation of cytotoxic cells involved in the elimination of infected cells from peripheral cirulation. Absence of TCR-Vβ12 mRNA expression during the course of infection requires further investigation to fully understand immune responses.

PATIENTS AND METHODS

Asymptomatic individuals

In a previous study, 7 of 24 exposed individuals were shown to be clearly infected by EBOV [14]. These individuals were family members of symptomatic patients who cared for them with no physical protection such as gloves. They were directly exposed to infected materials from fatal and non fatal cases such as faeces, vomit, sweat and blood. These asymptomatic individuals were sampled 2, 3 or 4 times after the first exposure to a sick patient. Plasma was analysed in all 7 individuals, and RT-PCR was performed on samples from 3 of them. All samples (collected 7, 9, 16 and 23 days after the initial exposure) were obtained, treated and stored in exactly the same conditions.

Detection of EBOV-specific IgG subclasses

We used an ELISA IgG assay, developed at CIRMF, in which Ebo-Z antigens or lysates from normal cultured Vero E6 cells are applied to the microtiter plates overnight at 4°C before washing with phosphate buffer saline (PBS) containing 0·1% Tween 20. The plates are saturated in PBS containing 3% bovine serum albumin (BSA) for 2 h at room temperature. Sera are diluted 1 : 25 in PBS containing 0·1% Tween 20, and 100 μl of each serum is dispensed in duplicate wells and left overnight at 4°C. After washing, the plates are incubated with biotin-conjugated mouse antibodies against human IgG subclasses (Sigma, l'Isle d'Abeau, France) for 2 h at room temperature. Binding is then revealed with streptavidin-peroxidase, and the TMB detector system is added (Dynex Technologies, Issy-les-Moulineaux, France). Optical density is measured at 492 nm, on an ELISA plate reader (Diagnostics Pasteur, Marnes la Coquette, France). The reagents were kindly provided by the Special Pathogens Branch, CDC (Atlanta, GA, USA). All specimens were handled according to WHO guidelines on viral haemorrhagic fever agents in Africa (Recommendations for management of viral haemorrhagic fevers in Africa, in *Workshop on Viral Haemorrhagic Fevers,* Sierra Leone, 1985).

Detection of EBOV RNA by RT-PCR

PBMC were separated from whole blood by Ficoll-diatrizoate density gradient centrifugation. Total RNA was extracted with a kit (Qiagen, Courtabœuf, France). The first-strand cDNA was synthesized using the Superscript II kit (Gibco BRL, Eragny, France), a dNTP mix (Amersham-Pharmacia Biotech, Orsay, France), and random hexamer primers (Boehringer, Mannheim, Germany). Half the reaction product was used as a template for PCR with Taq DNA polymerase (Appligene-Oncor, Illkirch, France) for 40 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 90 s). The reaction was run in a Perkin-Elmer 480 thermocycler (Perkin Elmer, Rotkreuz, Switzerland) with primers designed from a conserved region of the L gene, as described elsewhere [15]. The amplification products were analysed on 1·5% agarose gels. For nested PCR, 5 μl of the first-round reaction product was used as template, and the reaction was run in the same conditions as first-round PCR. The 298-bp products from nested PCR were also run in 1·5% agarose gel.

Detection of cytokines and soluble receptors in plasma

Plasma levels of IL-1RA, sIL6R, IL-12, IL-13, sTNFRI and, sTNFRII were measured with commercial kits (R & D Systems Europe, Abingdon, UK). Plasma levels of IL-10 and IFNγ were each measured simultaneously with two different kits (IL-10: Amersham-Pharmacia Biotech and Immunotech, Marseille, France; IFNγ: R & D Systems Europe and Immunotech). Plasma levels of the other cytokines were measured by using a two-site ELISA with specific capture and biotinylated antibodies against IL-2 or IL-6 (Genzyme, Cergy-Saint Christophe, France), IL-4, IL-5 or IL-10 (Pharmingen, CA, USA). To confirm the results, plasma levels of IL-2, IL-4 and IL-6 were also measured with commercial kits (Immunotech). Optical density was measured at 492 nm on an ELISA plate reader (Diagnostics Pasteur). Cytokines levels were calculated from standard curves. All samples were collected, treated and stored in exactly the same conditions.

Nuclear matrix protein ELISA

Plasma 41/7 NMP (Nuclear Matrix Protein) was measured with an ELISA kit (Calbiochem, Meudon, France) according the manufacturer's instructions.

Cortisol ELISA

Plasma cortisol was measured with the Bio-Mérieux cortisol diagnostic kit (Bio-Mérieux, Craponne, France) according the manufacturer's instructions.

RNA extraction and RT-PCR analysis of cytokine, activation markers and Vβ mRNA

PBMC were separated from whole blood as described above. mRNA was purified with a kit (Amersham-Pharmacia Biotech) from total RNA extracted as described above. mRNA was then amplified in a modified standard RT-PCR amplification procedure. The first-strand cDNA was synthesized as described using oligo$(dT)_{15}$ primers (Boehringer); one tenth of this reaction product was used as a template for PCR amplification. β-actin mRNA was used as a PCR control. Primers specific for cytokines, activation markers and TCR V_β genes were prepared by Genset (Paris, France). Primer sequences and annealing temperatures for cytokines and activation markers are shown in Table 1.

Analysis of the T-cell antigen receptor repertoire

RNA extraction and cDNA construction were performed as described above (see *Cytokine and Activation Markers*). PCR analysis of the different Vβs was based on a 5′-specific Vβ primer and a common 3′ constant-domain C_β primer as described [16]. Primer sequences for TCR V_β genes to 20 V_β gene sequences encompassing 18 V_β subgroups were as reported previously [17], namely V_β 1, 2, 3, 4, 5·1, 5·2, 6, 7, 8, 9, 10, 11, 12, 13·1, 14, 15, 16, 18, 19 and, 20.

Table 1 Primer sequences annealing temperatures and lengths of fragments obtained during RT-PCR analysis.

Genes	Primers (5' to 3')	T (°C)	fragment (bp)
Actine	CAGGCACCAGGGCGTGAT GCCAGCCAGGTCCAGACG	62	500
IL-2	TACAACTGGAGCATTTACTG GTTTCAGATCCCTTTAGTTC	50	268
IL-4	GCCTCACAGAGCAGAAGACT TCAGCTCGAACACTTTGAAT	52	344
IL-5	CTGATAGCCAATGAGACTCT TATTATCCACTCGGTGTTCA	50	250
IL-10	TGCCT–GGTCCTCCTGACTGG GCCTTGCTCTTGTTTCACA	60	389
IL-12	CGGATGCCCCTGGAGAAATG CTCTTGCCCTGGACCTGAAC	60	727
IFNγ	TGGAAAGAGGAGAGTGAC ATTGCTTTGCGTTGGACA	60	224
CD28	TTTCCTGTACAGGCCAAGTCT TTTGAGGGATCCCTCATTTGA	55	467
CTLA4	TTTCCGCCTATTTTCAGTTTA AGCATTTTGGTTTTGTTTTTC	55	484
CD40	CTGCCACCAGCACAAATACTG GGGTCCTCCTACCGTTTCTCT	55	223
CD40L	CAAACTTCTCCCCGATCTGC AATGGAGCTTGACTCGAAGC	56	548
Perf	CTACAGTTTCCATGTGGTAC TTATTGTCCCACACGGTGCT	50	600
Fas	GGGATTGGAATTGAGGAAGAC TTGGTGTTGCTGGTGAGTGTG	55	284
Fas-L	GATGGAGGGGAAGATGATGAG ATGCTGTGTTAGGAATGAAAT	55	750
CD4	TCCTTCCCACTCGCCTTTACA AGCACCCACACCGCCTTCTCC	58	500
CD8	CCCACTTTGTAGCCCCATCAC TCAGTCCTCCAGCACACTCTG	58	300

RESULTS

Kinetics of EBOV RNA during human asymptomatic infection

In samples taken on four occasions over a one-month period, starting about one week after initial exposure to the virus, we detected EBOV RNA in white blood cells by using a nested RT-PCR assay. The virus signal was present for up to two weeks after the last potential contact, i.e. three weeks after the initial contact, in at least two subjects (Fig. 1). Although viral RNA in PBMC is not an appropriate index of viral load, it is a reliable marker of infected cells.

IgG subclass responses during human asymptomatic Ebola infection

We have previously shown that EBOV-specific IgG starts to emerge 3–4 weeks after initial exposure and reaches moderate titres 1 month later. We thus evaluated EBOV-specific IgG subclasses at the highest levels time-point. As no standard sera for evaluation of EBOV-specific IgG subclasses was available, we determined the ratio as OD obtained when plates were coated with EBOV antigens over OD obtained when plates were coated with Vero-E6 cell lysates (Table 2). This ratio was 1 for each subclass in all negative endemic controls tested.

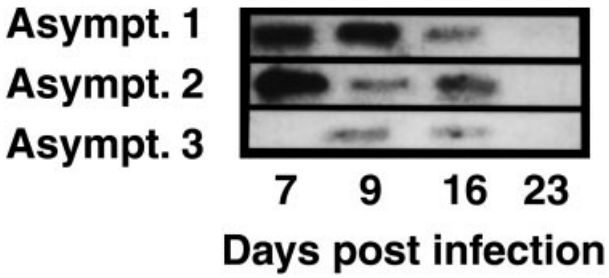

Fig. 1. Agarose gel electrophoresis of PCR DNA products from nested RT-PCR during the course of infection in 3 asymptomatic individuals. PBMC were collected from asymptomatic subjects 7, 9, 16 and 23 days after initial exposure to a sick patient. To control for possible RNA or DNA contamination, reactions without cDNA, and 10 negative controls were run in parallel (not shown).

IgG4 was never found in samples from asymptomatic subjects. IgG2 was detected in one subject and IgG1 in 3 subjects. The highest IgG1 level was observed in the only subject who had detected IgG2. IgG3 was found in 5/6 subjects. Again the highest IgG3 level was detected in the subject with IgG2. These data indicate that the humoral response is mainly IgG3/IgG1. Maturation of the humoral response seems to correspond to the emergence of IgG1 or IgG3 and after IgG2.

Inflammatory responses

We have previously shown that in subjects with asymptomatic EBOV infection the highest value observed of inflammatory responses was in the first sample studied that is around 7 days after the initial exposure, and disappear completely 2–3 days later [14]. In this previous study, levels of all pro-inflammatory cytokines tested (IL-1β, TNFα, IL-6, MCP and, MIP-1α/β) fell to normal within two to three days of their initial observation [14]. To understand this phenomenon, we assayed naturally circulating antagonists and two potent anti-inflammatory mediators, IL-10 and plasma cortisol (Fig. 2).

The IL-1 receptor antagonist (IL-1RA) and the two soluble TNF receptors (sTNFRI and sTNFRII) were found at moderate to high concentrations (3–7 ng/ml IL-1RA *versus* 1·6 ng/ml in 10 endemic controls; 1·3–2·6 ng/ml sTNFRI *versus* 1·8 ng/ml; 3·5–7 ng/ml sTNFRII *versus* 5 ng/ml) 7 days after initial exposure. Values fell two days later to normal endemic control levels, simultaneously with the fall in pro-inflammatory cytokines.

High plasma IL-10 concentrations (100–200 pg/ml *versus* $<$ 20 pg/ml in 10 endemic controls) and moderate plasma cortisol concentrations (200–400 ng/ml *versus* 92 ± 44 ng/ml in 10 endemic controls) were also found in these 7 asymptomatic subjects, and ran parallel to levels of circulating antagonists (Fig. 2).

Cytokine levels in plasma

No T-cell-derived cytokines (IL-2, IL-4, IL-5, IL-12 and, IFNγ) were detected in the plasma of the 7 asymptomatic subjects at any time.

T-cell responses (RT-PCR analysis)

We measured mRNA expression of various cytokines in PBMC from three patients by means of RT-PCR. Cytokine mRNA expression was normalized to β-actin mRNA. IL-2, IL-4 and

Table 2 IgG subclass responses to EBOV antigens. Positive total serum IgG from asymptomatic individuals (sampled 60 days after first exposure to infection) were tested for EBOV-specific IgG1, IgG2, IgG3 and IgG4 in an ELISA method. Each value represents the optical density (OD) ratio between sera reacted with EBOV antigens and with Vero-E6 cell lysates. This ratio was 1 in the 10 endemic controls tested (not shown). The absolute OD is shown in brackets

Patients	IgG1	IgG2	IgG3	IgG4
A	1	1	2.11(0.97)	1
B	1	1	2.53 (1.05)	1
C	2.82 (0.48)	1	1	1
D	1	1	4.15 (1.37)	1
E	6(1.56)	3.68 (1.25)	3.7 (1.23)	1
F	2.5 (1.04)	1	2.80 (0.48)	1

IL-10 and IL-12 showed identical mRNA expression profiles, peaking 9 days after initial exposure (i.e. at the end of the inflammatory process), and falling below the detection limit 7 days later (Fig. 3). RNA expression of several T-cell activation markers (CD28, CD40L and CTLA4) followed the same pattern as that of the cytokines, confirming T cell activation (Fig. 3). Cytokine and T cell activation markers mRNA was never detected in PBMC from endemic controls (Fig. 3).

To further characterize the consequences of T-cell activation, cytotoxic markers known to be involved in antiviral responses were studied in the same three asymptomatic individuals. Expression of IFNγ mRNA increased at the same time as the other cytokines (i.e. 9 days after initial exposure), but only declined in the final samples (more than 3 weeks after initial exposure) (Fig. 4a). RNA expression of Fas, FasL and, to a lesser extent, perforin followed a pattern identical to that of IFNγ, showing sustained, enhanced expression over the 3-week period after initial exposure (Fig. 4a) pointing to activation of cytotoxic cells and suggesting apoptosis.

To confirm this, we measured human nuclear matrix protein 41/7 (NMP), which is selectively released during apoptosis *in vitro* [18]. An increase in NMP was observed in plasma samples of 5 asymptomatic individuals, coinciding with down-regulation of cytotoxicity markers (Fig. 4b).

We then attempted to identify the cells involved in this cytotoxicity by analysing CD4 and CD8 mRNA expression by means of RT-PCR. In contrast to CD4, expression of CD8 mRNA was markedly elevated throughout the study period; CD8 mRNA levels started to fall at the last sampling time, again coinciding with the peak of NMP and with down-regulation of Fas/FasL mRNA expression (Fig. 4b).

Vβ repertoire during asymptomatic Ebola infection (RT-PCR analysis)

We then analysed the expression of 20 TCR-Vβ genes in PBMC

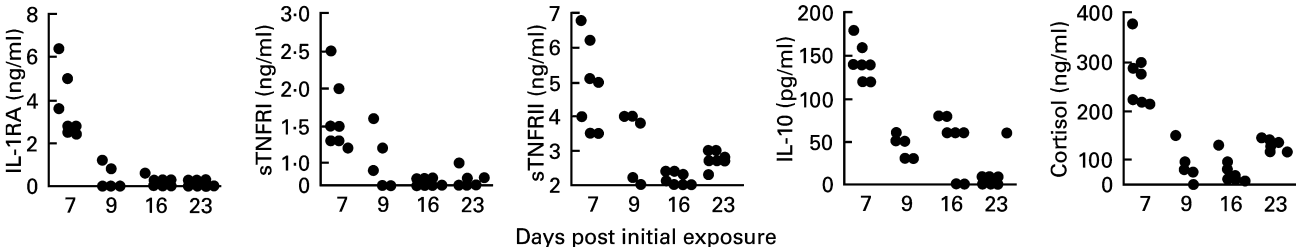

Fig. 2. Anti-inflammatory response. Naturally circulating antagonists (IL-1RA, sTNFRI, sTNFRII in ng/ml), IL-10 (in pg/ml), and plasma cortisol (in ng/ml) were measured as described in Methods. Individual values are shown 7, 9, 16 and 23 days following initial exposure to the virus. Each asymptomatic individual is represented by a point.

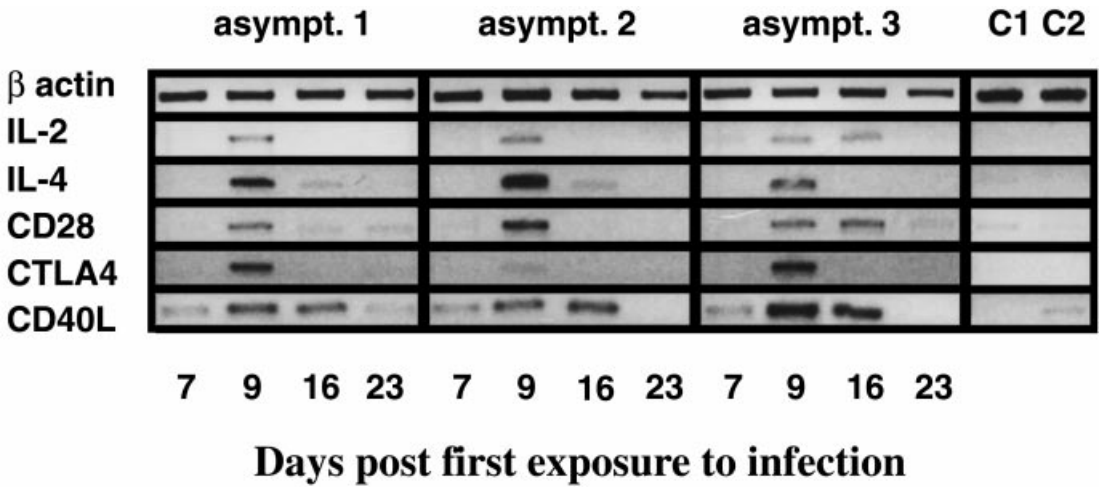

Fig. 3. T-cell responses. RNA expression of Th1 and Th2 cytokines (IL-2 and IL-4) and, T-cell activation markers (CD28, CD40L and CTLA4) was analysed as described in Methods. The results (ethidium bromide staining after electrophoresis on agarose gel) show the kinetics of mRNA expression in three asymptomatic individuals, 7, 9, 15 and 23 following initial exposure, and in two endemic controls.

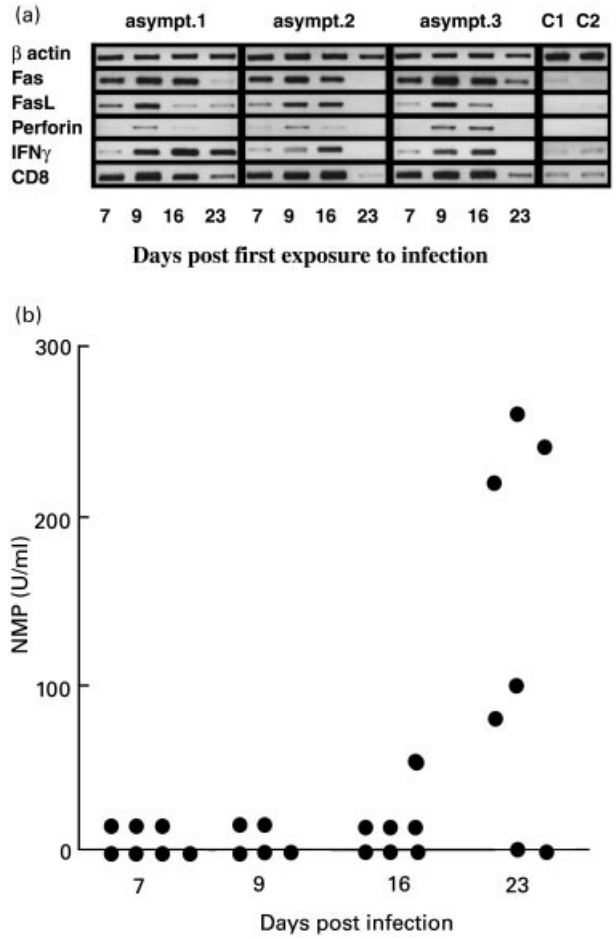

Fig. 4. Cytotoxic responses. (a) RNA expression of cytotoxicity markers (perforin, Fas, FasL, IFNγ and CD8) was analysed as described in Methods. The results (ethidium bromide staining after electrophoresis on agarose gel) show the kinetics of mRNA expression in three asymptomatic individuals, 7, 9, 15 and 23 following initial exposure, and in two endemic controls (b) Plasma 41/7 levels (Units/ml) were measured with an ELISA method. Each asymptomatic subject is represented by a point.

during the course of asymptomatic Ebola infection in 3 individuals. Expression of all 20 TCR-Vβ mRNAs was up-regulated in the 2 endemic controls tested (Fig. 5). By contrast, Vβ12 mRNA was only detected in one of the three asymptomatically infected subjects, and only at the last sampling time. RNA expression of the other TCR-Vβ species ran roughly parallel to CD8 mRNA expression, with sustained expression over the 3-week study period, followed by terminal down-regulation.

DISCUSSION

In a previous study we demonstrated that humans could be infected with Ebola virus without developing symptoms. These asymptomatic individuals produced specific IgM and IgG, starting between 2 and 3 weeks after initial exposure to infected body materials and reaching only moderate levels one month later. We also showed that they mounted a strong inflammatory response early in the infectious process characterized by high levels of IL-1β, TNFα, IL-6, MCP, MIP-1α and MIP-1β.

EBOV RNA was detectable in these subjects for up to three weeks after initial exposure. We therefore examined the cell-mediated response by ELISA testing of plasma and RT-PCR analysis of peripheral blood mononuclear cells (PBMC). We found that the inflammatory response coincided with anti-inflammatory processes, characterized by moderate levels of soluble antagonist (IL-1RA) and circulating TNF receptors (sTNFRI and sTNFRII), and high levels of potent anti-inflammatory mediators such as IL-10 and cortisol. The down-regulation of inflammatory responses was followed by transient T-cell activation for a few days, characterized by mRNA up-regulation of T-cell cytokines (IL-2 and IL-4) and T-cell activation markers (CD28, CD40L and CTLA4). This T-cell response was associated with cytotoxic cell activation characterized by marked and sustained up-regulation of IFNγ, FasL and perforin mRNA expression lasting for a further week. Disappearance of cytotoxic marker mRNAs three weeks after initial exposure coincided with the release of the apoptotic marker 41/7 NMP in plasma and the disappearance of viral RNA, suggesting that cytotoxic cells may clear infected cells from blood. This T-cell activation is probably

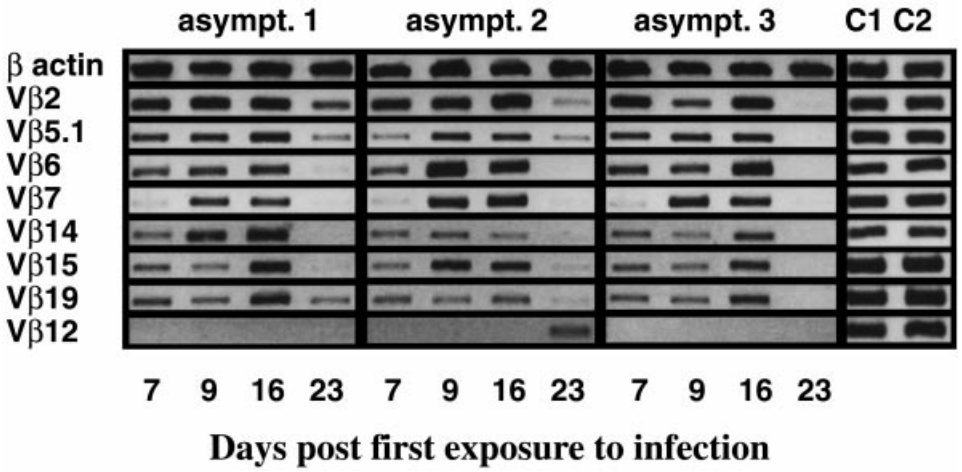

Fig. 5. Analysis of the T-cell antigen receptor-Vβ repertoire during asymptomatic Ebola infection by RT-PCR analysis. Agarose gel electrophoresis of PCR products for various ($n = 20$) combinations of Vβ gene family specific and Cβ oligonucleotide primers. Results show the kinetics of mRNA expression of six TCR-Vβ species in three asymptomatic individuals, 7, 9, 15 and 23 days following initial exposure, and in two endemic controls.

involved in production of EBOV-specific IgG3 and IgG1 subclasses, which are thought to play a role in limiting EBOV spread from 'privileged sites' such as semen.

Previously, we have shown that the inflammatory response in asymptomatic individuals is rapidly down-regulated [14]. All pro-inflammatory cytokines fell sharply to normal values within 2–3 days of their initial detection. Three factors may account for this rapid resolution of inflammation. Firstly, the short-lives of TNF, IL-1β and IL-6 may account in part for their rapid disappearance. Secondly, the simultaneous occurrence of naturally circulating antagonists such as the IL-1 receptor antagonist (IL-1RA) and the two soluble TNF receptors (sTNFRI, 55 kD, and sTNFRII, 75 kD) may participate in the down-regulation of inflammatory responses. IL-1RA has been shown to inhibit the inflammatory action of IL-1β by competitive binding to its natural receptor [19–21], and soluble TNF receptors I and II bind directly to TNF, thus participating in the neutralization and clearance of TNF [22–24]. Thirdly, high levels of IL-10 and cortisol, found in the plasma of asymptomatic individuals during the early stages of the infectious process, may also participate in dampening the inflammatory response. IL-10 [25–27] and cortisol [28,29] have been shown to potently inhibit pro-inflammatory cytokine and chemokine production by monocytes and to stimulate production of the soluble circulating inhibitors sTNFRs and IL-1RA [30,31]. These observations are similar to those made in acute meningococcal infection, where high levels of pro-inflammatory cytokines are modulated by early simultaneous production of anti-inflammatory cytokines [32,33]. Similarly, pro-inflammatory and anti-inflammatory cytokines are simultaneously secreted during other forms of haemorrhagic fever. For instance, elevated plasma levels of TNF, sTNFRI/II and IL-10 are detected simultaneously in patients with haemorrhagic fever with renal syndrome [34]. High levels of IFNα, TNF, IL-6, IL-8 and IL-10 are present at the same time in patients with Argentine haemorrhagic fever [35]. Taken together, these observations suggest that pro- and anti-inflammatory components form an autoregulatory loop, in which IL-10 and cortisol down-regulate pro-inflammatory cytokines and stimulate their antagonists, and sTNFRI/II and IL-1RA reduce the bioactivity of inflammatory cytokines. Thus, the rapid down-regulation of inflammatory responses in asymptomatic individuals presumably avoids excessive fever and tissue damage

Viral RNA was still detectable for up to two weeks after the down-regulation of the inflammatory response. We therefore examined the cell-mediated immune response. Expression of mRNA IL-2 and IL-4 was transiently increased around day nine after initial exposure, i.e. simultaneously with the down-regulation of the inflammatory process. Interestingly, this cytokine mRNA expression was associated with concomitant mRNA up-regulation of the CD28 and CD40L T cell activation markers. These data indicate that T cells are activated and that a transient but effective T cell-mediated response occurs in asymptomatic individuals. This is further reinforced by the increase in mRNA expression of CTLA4, which is known to be enhanced on the T-cell surface in the later stages of the T-cell activation cascade [36,37].

The consequences of T-cell activation were investigated by measuring mRNA expression of cytotoxicity markers known to be involved in antiviral responses. The marked and sustained increase in mRNA expression of IFNγ, the cytotoxicity markers Fas, FasL and, to a lesser extent, perforin indicated activation of cytotoxic T cells. Both Fas and perforin are known to mediate cytotoxic functions [38,39], and these two mechanisms of cell-mediated cytotoxicity, one based on the secretion of lytic proteins such as perforin, and the second involving cell-surface Fas/FasL interactions, have been shown to play a major role in many viral infections by directly killing infected cells [40]. It is well known that both mechanisms induce apoptosis of target cells. To confirm that programmed cell death occurred we measured human nuclear matrix protein 41/7 NMP, which is selectively released during apoptosis *in vitro* [18]. An increase in NMP was observed in the plasma of 5 of the 7 asymptomatic subjects, and coincided with the final down-regulation of cytotoxicity marker mRNA synthesis and the disappearance of virus RNA from PBMC. In contrast to CD4, expression of CD8 mRNA was markedly stimulated throughout the study period compared with endemic control subjects, and only decreased at the last time point, again coinciding with the peak in NMP and the down-regulation of Fas/FasL mRNA expression. These data indicate that CD8 T cells may be involved in cell-mediated cytotoxicity in this setting. As monocytes have been shown to undergo apoptosis induced by both perforin and Fas [41,42], and as monocytes are infected by filoviruses, we suggest that these two cytotoxic pathways may account for the elimination of EBOV-infected cells.

The second consequence of T-cell activation is the production of EBOV-specific IgG, which was detectable 3 weeks after initial exposure, i.e. a few days after T-cell activation. EBOV-specific IgG was predominantly composed of IgG3 and, to a lesser extent, IgG1. This IgG3/IgG1 pattern is consistent with previous investigations in which viral infections have been shown mainly to induce antibodies restricted to the IgG1 and IgG3 subclasses [43]. Viruses mainly induce T cell-dependent B cell responses. In this context, IgG production by B lymphocytes requires both interaction of B cell receptors with antigens and cognate interaction between B lymphocyte and activated T helper cells through CD40–CD40 ligand interaction [44]. The CD40–CD40L interaction and polar secretion of cytokines finally induce B cell proliferation [45], IgG production [46], and, later, isotype switching [44,47]. Because of the late emergence of specific IgG, the role of later humoral responses in limiting virus replication is unlikely but, given the neutralizing capacities of IgG1 and IgG3 in most viral infections [48], these later humoral responses may be involved in limiting Ebola virus from spread from 'privileged sites' such as semen where infectious virus has been found in patients several months after the illness [49,50].

Finally, RT-PCR analysis of the T-cell receptor (TCR)-Vβ repertoire in PBMC of patients who died showed a gradual disappearance of all TCR-Vβ mRNA species [13]. By studying the same TCR-Vβ by means of RT-PCR analysis in asymptomatic individuals infected with very small amounts of EBOV, we sought to determine whether the TCR-Vβ repertoire underwent particular modifications during the course of the infection in these individuals. The pattern of mRNA expression of all TCR-Vβ (except Vβ12) during the course of the infection globally ran parallel to that of CD8 mRNA, with sustained expression for 3 weeks followed by terminal down-regulation, suggesting that activated CD8 T cells are deleted by apoptosis. In contrast, mRNA expression of TCR-Vβ12 was never detected during the course of the infection, confirming data obtained in fatal and non fatal cases (Baize & Leroy, unpublished observation), and indicating either an anergic state or deletion of this Vβ12 species. Because we were unable to show whether Vβ12 mRNA expression was markedly up-regulated in the initial stage of the infection, and

because of the limitations of the RT-PCR technique, it is possible that the specific disappearance of the mRNA signal was due to a superantigen effect of EBOV or to random deletion or anergy of one of the minor Vβ species in the TCR-Vβ repertoire. In vitro studies at the protein level are required. The onset of Vβ12 mRNA synthesis in PBMC in one asymptomatic subject at the latest time-point probably represents a return to normal immunological status, and the clearance of EBOV RNA from the peripheral circulation.

In conclusion, we show that 7 individuals exposed to Ebola virus during outbreaks in Gabon were infected by the virus but never developed symptoms of acute disease nor antigenemia detectable by antigen-capture ELISA. However, Ebola virus RNA was present in the 3 individuals studied for up to 3 weeks following exposure. Virus replication and disease onset were apparently controlled by an inflammatory response, which was subsequently down-regulated by an anti-inflammatory response. Cytotoxic mechanisms appeared to be involved in the clearance of virus-infected cells. Finally, the absence of TCR-Vβ12 mRNA expression, contrary to 19 other TCR-Vβ species during the course of the infection, suggests preferential dysregulation of T-lymphocyte subpopulations expressing the Vβ12 gene; this phenomenon requires further investigation to elucidate the primary immune response to EBOV infection.

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REFERENCES

1 Feldmann H, Klenk HD. Marburg and Ebola viruses. Adv Virus Res 1996; **47**:1–52.

2 Feldmann H, Klenk HD, Sanchez A. Molecular biology and evolution of filoviruses. Arch Virol Supplement 1993; **7**:81–100.

3 Ksiazek TG, Rollin PE, Williams AJ *et al.* Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic Congo, 1995. J Infect Dis 1999; **179**:S177–87.

4 Connolly BM, Steele KE, Davis KJ *et al.* Pathogenesis of experimental Ebola virus infection in Guinea pigs. J Infect Dis 1999; **179**:S203–S217.

5 Fisher-Hoch SP, McCormick JB. Current Topics in Microbiology and Immunolgy: Marburg and Ebola Viruses, Berlin: Springer-Verlag, 1999.

6 Fisher-Hoch SP, Brammer TL, Trappier SG *et al.* Pathogenic potential of filoviruses: Role of geographic origin of primate host and virus strain. J Infect Dis 1992; **166**:753–63.

7 Sadek RF, Khan AS, Stevens G *et al.* Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995 Determinants of survival. J Infect Dis 1999; **179**:S24–S27.

8 Jahrling PB, Geisbert TW, Geisbert JB *et al.* Evaluation of immune globulin and recombinant interferon-α2b for treatment of experimental Ebola virus infections. J Infect Dis 1999; **179**:S224–S234.

9 Feldmann H, Bugany H, Mahner F *et al.* Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. J Clin Invest 1996; **70**:2208–14.

10 Geisbert TW, Jahrling PB, Hanes MA *et al.* Association of Ebola-related Reston virus particles and antigen with tissue lesions of monkeys imported to the United States. J Comp Pathol 1992; **106**:137–52.

11 Ryabchikova E, Kolesnikova L, Smolina M *et al.* Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis. Arch Virol 1996; **141**:909–21.

12 Bray M, Davis K, Geisbert T *et al.* A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis 1998; **178**:651–61.

13 Baize S, Leroy EM, Georges-Courbot M-C *et al.* Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. Nature Med 1999; **5**:1–5.

14 Leroy EM, Baize S, Volchkov VE *et al.* Human asymptomatic Ebola infection exists and is associated with a strong inflammatory response. Lancet 2000; **355**:2210–5.

15 Sanchez A, Ksiazek TG, Rollin PE *et al.* Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J Infect Dis 1999; **179**:S164–9.

16 Cottrez F, Auriault C, Capron A *et al.* Analysis of the Vβ specificity of superantigen activation with a rapid and sensitive method using RT PVR and an automatic DNA analyser. J Immunol Methods 1994; **172**:85–94.

17 Imberti L, Sottini A, Bettinardi A *et al.* Selective depletion in HIV infection of T cells that bear specific T cell receptor Vβ sequences. Science 1991; **254**:860–2.

18 Arguello F, Sterry JA, Zhao YZ *et al.* Two serologic markers to monitor the engrafment, growth, and treatment response of human leukemias in severe combined immunodeficient mice. Blood 1996; **87**:4325–32.

19 Ohlsson K, Björk P, Bergenfeldt M *et al.* Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. Nature 1990; **348**:550–2.

20 Hannum CH, Wilcox CJ, Arend WP *et al.* Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. Nature 1990; **343**:336–40.

21 Arend WP, Malyad M, Guthridge CJ *et al.* Interleukin-1 receptor antagonist: role in biology. Ann Rev Immunol 1998; **16**:27–55.

22 Kohno T, Brewer MT, Baker SL *et al.* A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor. Proc Natl Acad Sci USA 1990; **87**:8331–5.

23 van Zee KJ, Kohno T, Fischer E *et al.* Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor a *in vitro* and *in vivo*. Proc Nat Acad Sci USA 1992; **89**:4845–9.

24 Aderka D, Engelmann H, Maor Y *et al.* Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med 1992; **175**:323–9.

25 de Waal Malefyt R, Abrams J, Bennett B *et al.* Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991; **174**:1209–20.

26 Brandtzaeg P, Osnes L, Ovstebo R *et al.* Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: identification of interleukin-10 as a major functional deactivator of human monocytes. J Exp Med 1996; **184**:51–60.

27 Olszyna DP, Pajkrt D, Lauw FN *et al.* Interleukin 10 Inhibits the Release of CC Chemokines during Human Endotoxemia. J Infect Dis 2000; **181**:613–20.

28 Zuckerman SH, Shellhaas J, Butler LD. Differential regulation of lipopolysaccharide-induced interleukin 1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. Eur J Immunol 1989; **19**:301–5.

29 Goujon E, Parnet P, Laye S *et al.* Adrenalectomy enhances pro-inflammatory cytokines gene expression, in the spleen, pituitary and brain of mice in response to lipopolysaccharide. Mol Brain Res 1996; **36**:53–62.

30 Joyce DA, Gibbons DP, Green P *et al.* Two inhibitors of pro-inflammatory cytokine release, interleukin-10 and interleukin-4, have contrasting effects on release of soluble p75 tumor necrosis factor receptor by cultured monocytes. Eur J Immunol 1994; **24**:2699–705.
31 Seitz M, Loetscher P, Dewald B *et al.* Interleukin-10 differentially regulates cytokine inhibitor and chemokine release from blood mononuclear cells and fibroblasts. Eur J Immunol 1995; **25**:1129–32.
32 van Deuren M, van der Ven-Jongekrijg J, Bartelink AKM *et al.* Correlation between proinflammatory cytokines and antiinflammatory mediators and the severity of disease in meningococcal infections. J Infect Dis 1995; **172**:433–9.
33 van Deuren M, van der Ven-Jongekrijg J, Vannier E *et al.* The pattern of Interleukin-1β (IL-1β) and its modulating agents IL-1 receptor antagonist and IL-1 soluble receptor type II in acute meningococcal infections. Blood 1997; **90**:1101–8.
34 Linderholm M, Ahlm C, Settergren B *et al.* Elevated plasma levels of Tumor Necrosis Factor (TNF)-α, soluble TNF receptors, interleukin (IL) -6, and IL-10 in patients with hemorrhagic fever with renal syndrome. J Infect Dis 1996; **173**:38–43.
35 Marta RF, Montero VS, Hack CE *et al.* Proinflammatory cytokines and elastase-alpha-1-antitrypsin in Argentine hemorrhagic fever. Am J Trop Med Hyg 1999; **60**:85–9.
36 Hathcock KS, Laszio G, Dickler HB *et al.* Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science 1993; **262**:905–9.
37 Brunet J-B, Denizot F, Luciani M-F *et al.* A new member of the immunoglobulin superfamily-CTLA-4. Nature 1987; **328**:267–70.
38 Kägi D, Vignaux F, Ledermann B *et al.* Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 1994; **265**:528–30.
39 Lowin B, Hahne M, Mattmann C *et al.* Cytolytic T-cell cytotoxicity is mediated through perforin and fas lytic pathways. Nature 1994; **370**:650–2.
40 Kägi D, Seiler P, Pavlovic J *et al.* The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. Eur J Immunol 1995; **25**:3256–62.
41 Richardson BC, Lalwani ND, Johnson KJ *et al.* Fas ligation triggers apoptosis in macrophages but not endothelial cells. Eur J Immunol. 24:1994.
42 Kiener PA, Davis PM, Starling GC *et al.* Differential induction of apoptosis by Fas–Fas ligand interactions in human monocytes and macrophages. J Exp Med 1997; **185**:1511–6.
43 Linde A, Sundqist VA, Mathiesen T *et al.* IgG subclasses to subviral components. Monogr Allergy 1988; **23**:27–32.
44 Parker DCT. cell-dependent B cell activation. Ann Rev Immunol 1993; **11**:331–60.
45 Durie FH, Foy TM, Masters SR *et al.* The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol Today. 1994; **15**:406–410.
46 Allen RC, Armitage RJ, Conley ME *et al.* CD40 ligand gene defects responsible for X-linked hyper–IgM syndrome. Science 1993; **259**:990–3.
47 Van den Eertwegh AJM, Noelle RJ, Roy M *et al.* In vivo CD40–gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T–B cell interactions. J Exp Med 1993; **178**:1555–65.
48 Gilljam G, Wahren B. Properties of IgG subclasses to human cytomegalovirus. J Virol Methods 1989; **25**:139–51.
49 Martini GA, Siegert R. Marburg virus disease. New York: Springer, 1971.
50 Emond RT, Evans B, Bowen ET *et al.* A case of Ebola virus infection. Br Med J 1977; **2**:541–4.